THE PHYSIOLOGY OF INSECT DIAPAUSE. VIII. QUALITATIVE CHANGES IN THE METABOLISM OF THE CECROPIA SILKWORM DURING DIAPAUSE AND DEVELOPMENT [1]

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In the Cecropia silkworm the termination of pupal diapause and the progress of adult development are accompanied by large and predictable changes in respiratory metabolism. Thus, as described in the preceding paper of this series, the respiration of Cecropia midway its adult development is approximately seven times that of the diapausing pupa. In the present study efforts were made to ascertain the enzymatic basis of the quantitative changes in respiration. Considerable evidence was already at hand pointing to pronounced alterations in the cytochrome system in synchrony with the termination of diapause in the eggs of the grasshopper *Melanoplus* (Bodine and Boell, 1934a, 1934b), the eggs of the commercial silkworm *Bombyx* (Wolsky, 1943), and the pupa of the Cecropia silkworm (Sanborn and Williams, 1950; Pappenheimer and Williams, 1952; Schneiderman and Williams, 1952). For this reason attention focussed on the role of the terminal oxidases in relation to diapause and development.

The principal terminal oxidases which have been demonstrated in animals and higher plants are cytochrome oxidase, flavoproteins, and copper-containing proteins such as ascorbic acid oxidase and tyrosinase (Lardy, 1949; Goddard and Meeuse, 1950). Among these, all save ascorbic acid oxidase are thought to function as terminal oxidases in certain animal cells, though the precise role which tyrosinase may play has never been satisfactorily defined (Sussman, 1949).

In animals such as insects, when hemoglobin and other erythrocruorins are absent, carbon monoxide inhibits cytochrome oxidase and tyrosinase (Warburg, 1949), but apparently fails to inhibit flavoproteins or any other enzymes or substrates. It is true that carbon monoxide forms spectroscopically identifiable complexes with certain peroxidases, but peroxidase activity remains uninhibited (Theorell, 1953). Carbon monoxide's inhibition of cytochrome oxidase and tyrosinase can be distinguished in that the former is reversed by light while the latter is not (Warburg and Negelein, 1928; Kubowitz, 1937, 1938). Carbon monoxide therefore affords a remarkably specific tool for tracking the participation of the cytochrome oxidase system in biological reactions. In the present study we have exploited this specificity in an effort to characterize the terminal oxidases of the Cecropia silkworm during diapause and development.

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For reasons considered elsewhere (Schneiderman and Feder, 1954), the effects of high concentrations of carbon monoxide were studied by positive pressure techniques. Animals were placed in transparent, air-filled, polymethyl methacrylate (Lucite) chambers, compressed with carbon monoxide, and measurements of respiration performed by means of respirometers developed for use at high pressures. Under these conditions the oxygen tension remained unchanged at its normal value of one-fifth of an atmosphere, while the carbon monoxide pressure could be increased to as high as seven atmospheres. The positive pressure techniques were supplemented by experiments performed at atmospheric pressure and testing the effects of carbon monoxide, oxygen tension, and cyanide.

Materials and Methods

Diapausing pupae and developing adults of the giant silkworm, *Platysamia cecropia,* were used as experimental animals. In the case of the pupal material the brains were commonly removed to stabilize the animals in permanent diapause (Williams, 1946). In order to avoid the complication of "post-injury metabolism" (Schneiderman and Williams, 1953a), one month or longer was allowed for the recovery of animals subjected to surgical manipulation. All experiments were performed at 25° C.

1. *Metabolic studies*

In studies performed at atmospheric pressure the insects were placed in individual 45-cc. vessels of the type described previously (Schneiderman and Williams, 1953a), and the oxygen consumption determined by the Warburg method. Experiments at positive pressures were carried out in high pressure respirometers (Schneiderman and Feder, 1954). Measurements were begun approximately 1½ hours after compression and continued for 20 to 30 hours in order to compensate for the discontinuous release of carbon dioxide by diapausing pupae (Punt, 1950; Schneiderman and Williams, 1953a, 1953b). The gas volume of the individual respirometers was sufficiently large to preclude any important decrease in oxygen tension during the experimental period.

At the end of positive pressure experiments, acid was added to the alkali and the displaced carbon dioxide measured volumetrically (Schneiderman and Williams, 1953a). The total output of carbon dioxide was measured from the moment the respirometers were sealed to the moment the animals were removed, and the average carbon dioxide production estimated during this period. The over-all respiratory quotient for the duration of the experimental period was calculated from the average carbon dioxide production divided by the average oxygen consumption.

2. *Experimental gases*

The gases were obtained in commercial cylinders and assayed as follows:

Nitrogen (Airco), 99.5% N_2 plus less than 0.5% oxygen. Oxygen (Airco), 99.5% oxygen plus less than 0.5% nitrogen. Carbon monoxide (Matheson Co.), 96.8% carbon monoxide, 0.36% carbon dioxide, 0.97% hydrogen, 1% nitrogen, 0.8% saturated hydrocarbons, 1.19 mg. iron per liter, 0.32 mg. sulfur per liter.

Prior to its use, the carbon monoxide was bubbled through a solution of 10% sodium hydroxide to remove carbon dioxide and iron carbonyl compounds.

In one series of experiments, 200 liters of extremely pure carbon monoxide were prepared by the action of hot concentrated sulfuric acid (C. P. reagent) on formic acid (analytical reagent). The carbon monoxide was passed, in turn, through an aqueous solution containing 5% pyrogallic acid and 25% KOH, a dry ice-acetone trap, $CaCl_2$, $Mg(ClO_4)_2$ ("Anhydrone"), a liquid nitrogen trap, and then compressed to 100 psi in small steel cylinders. Since the effect of this pure carbon monoxide on respiration could not be distinguished from that of the alkali-treated commercial carbon monoxide, the less expensive commercial gas was used in subsequent experiments.

Mixtures of carbon monoxide, oxygen, and nitrogen were prepared under pressure in steel or Lucite tanks and their compositions checked by gas analysis (Scholander and Roughton, 1953).

3. *Cyanide experiments*

To appraise the metabolic effects of cyanide, the insects were first weighed and their water content assumed to equal 75 per cent of the live weight. Then, by means of an extremely small (30) gauge hypodermic needle, each pupa was injected just lateral to the midline of the thoracic tergum with 0.05 to 0.09 ml. of freshly prepared neutralized KCN. The latter's concentration was regulated to establish a specific final concentration after dilution with the fluid volume of the insect. At the pH of the insect, KCN exists almost wholly as HCN; the molar concentration of HCN within the insect was calculated on this basis.

Immediately after injection each pupa was enclosed in a Warburg vessel. As recommended by Robbie (1946), mixtures of KCN and KOH were placed in the vessel for the absorption of carbon dioxide; in this manner the HCN concentration of the chamber was balanced against the internal concentration established within the insect by the injection. At internal HCN concentrations of 10^{-3} M or greater, a constant external HCN tension of 5×10^{-4} was employed—the highest concentration that one can establish by means of KCN–KOH mixtures. In one series of experiments the experimental animals were equilibrated *via* the tracheal system with a specific tension of HCN for 60 hours and then studied without the actual injection of cyanide.

Experimental Results

1. *Effects of carbon monoxide at atmospheric pressure on the respiration of diapausing pupae*

In experiments performed on two diapausing pupae and five brainless diapausing pupae the rate of oxygen consumption was first measured in air, then in 6 per cent oxygen plus 94 per cent nitrogen, and, finally, in 6 per cent oxygen plus 94 per cent carbon monoxide (carbon monoxide/oxygen = 16:1). During the course of the experiment the respirometers were flushed periodically with the experimental gases to prevent the uptake of oxygen from appreciably diminishing the oxygen tension in the vessels. As recorded in Table I, it is evident that between the first and eighth hours of exposure to carbon monoxide only one pupa showed any appreciable inhi-

TABLE I

*The effects of carbon monoxide on the oxygen consumption of two diapausing and five brainless diapausing pupae**

Type of pupa	Rate of oxygen consumption (mm.³/gm. live wt./hr.)		Relative rate of oxygen consumption in 16:1 CO/O_2 (%)		
	Air	16:1 N_2/O_2	After 1 hour	Between 1st and 8th hour	Between 8th and 28th hour
Diapausing	9.0 (76%)	11.8 (100%)	126	87	—
Diapausing	11.5 (91%)	12.6 (100%)	132	132	—
Brainless diapausing	8.8 (106%)	8.3 (100%)	116	94	144
Brainless diapausing	9.7 (99%)	9.8 (100%)	85	94	85
Brainless diapausing	9.9 (89%)	11.1 (100%)	95	93	84
Brainless diapausing	11.8 (91%)	13.0 (100%)	106	98	89
Brainless diapausing	13.4 (106%)	12.7 (100%)	73	65	79

* All measurements performed at a total pressure of one atmosphere.

bition of respiration. Between the eighth and twenty-eighth hours four of the brainless diapausing pupae showed about 15 per cent inhibition.

These results demonstrate that only a small fraction of the metabolism of Cecropia pupae is carbon monoxide-sensitive when the carbon monoxide/oxygen ratio is 16:1. To test the effects of still higher ratios, a considerable number of experiments were performed making use of the positive pressure respirometers.

2. *Effects of high pressures of nitrogen on the respiration of brainless diapausing pupae*

Figure 1 illustrates results typical of a number of control experiments in which the respiration of five brainless diapausing pupae was determined in one atmosphere of air and then in air compressed with five atmospheres of nitrogen. It is evident that positive pressures of five atmospheres of an inert gas such as nitrogen were without notable effects on either the rate of oxygen consumption or carbon dioxide output. The slight increase in carbon dioxide output is most probably an artifact attributable to the flushing of stored carbon dioxide from the animal during the period of decompression (Schneiderman and Williams, 1953b).

3. *Effects of high pressures of carbon monoxide on the respiration of brainless diapausing pupae*

Brainless diapausing pupae were placed in air-filled respirometers and their respiration measured after compression with five atmospheres of nitrogen; they were then decompressed to air and the measurements repeated after recompression with five atmospheres of carbon monoxide (carbon monoxide/oxygen ratio of 25:1). Figure 2 records the results obtained in an experiment utilizing five brainless diapausing pupae. A comparison of the respiration in nitrogen and in carbon monoxide reveals that about one-third of the oxygen consumption was inhibited by the high pressure of carbon monoxide. Carbon dioxide production was affected to a lesser degree to yield an apparent increase in the respiratory quotient.

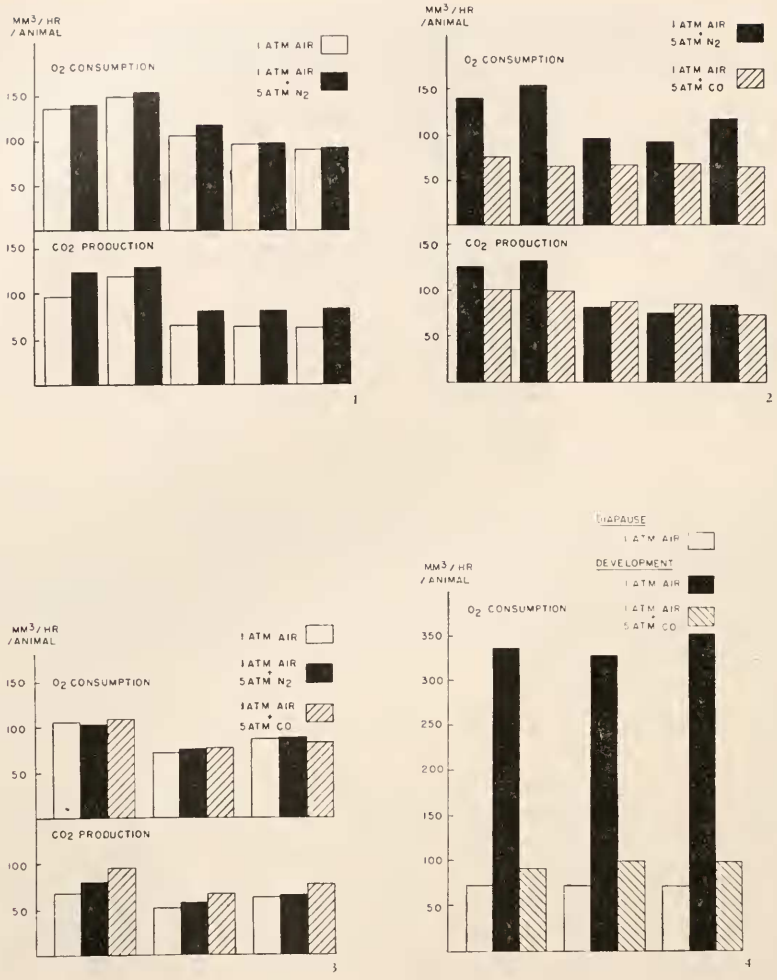

FIGURE 1. The average respiration of five brainless diapausing pupae in air at one atmosphere compared with the average respiration of the same animals in air compressed with 5 atmospheres of nitrogen.

FIGURE 2. The average respiration of five brainless diapausing pupae in air compressed with 5 atmospheres of nitrogen compared with the average respiration of the same animals in air compressed with 5 atmospheres of carbon monoxide.

FIGURE 3. The average respiration of three brainless diapausing pupae, lacking abdominal ganglia, in air at one atmosphere pressure, in air compressed with 5 atmospheres of nitrogen, and in air compressed with 5 atmospheres of carbon monoxide.

FIGURE 4. The average oyxgen consumption of three developing animals on the sixth day of adult development, in air at one atmosphere pressure, and in air compressed with 5 atmospheres of carbon monoxide. The normal average oxygen consumption of diapausing pupae in air at one atmosphere pressure is also recorded.

4. *Effects of high pressures of carbon monoxide on the respiration of brainless diapausing pupae lacking abdominal ganglia*

Taken at face value, experiments of the type just considered suggest that about one-third of the metabolism of diapausing pupae is mediated *via* the cytochrome oxidase system. However, it was noted that diapausing pupae showed a conspicuous depression in the frequency and amplitude of spontaneous muscular movements of the abdominal segments in the presence of high pressures of carbon monoxide. It seemed possible that the observed inhibition by carbon monoxide might arise from a suppression of these movements rather than from inhibition of the pupa as a whole.

This possibility was tested by a repetition of the preceding experiment on a series of brainless diapausing pupae in which the intersegmental muscles of the abdomen had previously been denervated by removal of the chain of eight abdominal ganglia. A total of four such animals were studied in detail. Figure 3 records the respiratory exchange of three of these individuals in air, in air compressed with five atmospheres of nitrogen, and in air compressed with five atmospheres of carbon monoxide. The rates of oxygen consumption under all three conditions were indistinguishable—a result which indicates that in the absence of muscular movements of the abdomen the metabolism of diapausing pupae is insensitive to carbon monoxide.

5. *Effects of carbon monoxide on pilocarpine-stimulated muscular activity and respiration*

It was known from studies to be considered elsewhere that the injection of suitable concentrations of pilocarpine causes diapausing pupae to move their abdomens continuously for up to a year thereafter. Consequently, animals stimulated in this manner afforded ideal material for testing the sensitivity of the abdominal motion and the accompanying respiration to inhibition by carbon monoxide. To this end, each of a series of nine diapausing pupae was injected with 0.1 ml. of 0.1 *M* pilocarpine hydrochloride that had previously been neutralized to pH 6.6 with sodium hydroxide. Two days later, the pupae were enclosed in an air-filled Lucite chamber, compressed with specific pressures of carbon monoxide or oxygen, and the effects on abdominal motion noted.

Carbon monoxide inhibited the abdominal motion to a degree dictated by the carbon monoxide/oxygen ratio. Thus, when a ratio of 10 : 1 was established by the addition of 30 psi carbon monoxide to the initial atmosphere of air, abdominal motion was markedly inhibited. When the ratio was then decreased to 3 : 1 by the addition of 7 psi of oxygen, vigorous movements reappeared. Further compression with carbon monoxide once again restored the inhibition. In virtually all cases abdominal motion ceased when the ratio was as high as 15 : 1, but was resumed within 10 minutes after the decompression and return to air.

Ten days after the experiment just considered the oxygen consumption of five of the continuously wriggling pupae was measured at atmospheric pressure in air, and in specific mixtures of oxygen, nitrogen, and carbon monoxide. The results summarized in Table II reveal that 16 : 1 carbon monoxide/oxygen caused a prompt cessation of the abdominal motion and inhibited the oxygen consumption by approximately 30 per cent.

TABLE II

*The effects of carbon monoxide on the oxygen consumption and abdominal motion of five diapausing pupae injected with 0.1 ml. of 0.1 M pilocarpine hydrochloride**

Rate of oxygen consumption (mm.³/animal/hour)		Relative rate of oxygen consumption in 16:1 CO/O_2 (%)	
Air	16:1 N_2/O_2	During 1st hour	During 3rd hour
81 (89%) (+)	91 (100%) (+)	52 (−)	52 (−)
86 (77%) (+)	112 (100%) (+)	90 (-)	87 (−)
113 (94%) (+)	120 (100%) (+)	58 (−)	58 (−)
133 (116%) (+)	115 (100%) (+)	81 (−)	81 (−)
179 (80%) (+)	223 (100%) (+)	75 (−)	75 (−)
Average: 118 (89%)	132 (100%) (+)	72 (−)	71 (−)

* All measurements performed at a total pressure of one atmosphere.
(+) Records the presence of abdominal motion; (−) the absence of same.

Taken along with the previously mentioned experiments, these findings provide a consistent body of evidence that the contraction of the intersegmental muscles of the diapausing pupa is inhibited by carbon monoxide, whereas the other tissues of the dormant insect are not inhibited by carbon monoxide.

6. *Effects of carbon monoxide on the increased respiration accompanying adult development*

After the termination of pupal diapause the onset and progress of adult development are accompanied by a rapid increase in respiration. Thus on the sixth day of adult development the average respiration is approximately five times that during diapause. In order to ascertain the carbon monoxide-sensitivity of this additional metabolism accompanying development, the respiration of four animals on the sixth day of adult development was first measured in air and then in air compressed with five atmospheres of carbon monoxide. The results, illustrated in the case of the three individuals in Figure 4, demonstrate a striking effect of this 25 : 1 carbon monoxide/oxygen ratio on the respiration of developing animals. About two thirds

TABLE III

*The effects of carbon monoxide on the oxygen consumption of developing adults**

Days after initiation of adult development	Rate of oxygen consumption (mm.³/gm. live wt./hr.)		Relative rate of oxygen consumption in 16:1 CO/O_2 (%)		
	Air	16:1 N_2/O_2	During 1st hour	During 4th hour	During 8th hour
2	54 (102%)	53 (100%)	68	56	55
5½	84 (129%)	65 (100%)	64	52	39
6½	99 (121%)	82 (100%)	68	56	54

* All experiments performed at a total pressure of one atmosphere.

of the oxygen consumption was inhibited and the metabolism dropped to a level almost as low as that of diapausing pupae. Consequently, it appears that the increased oxygen consumption accompanying adult development is completely or almost completely inhibited by carbon monoxide.

Table III records analogous findings in an experiment in which three developing adults were exposed to a mixture of carbon monoxide and oxygen at a total pressure of one atmosphere. To compensate for the utilization of oxygen, the Warburg vessels were reflushed with the experimental gas every 2½ hours. It will be noted that the 16:1 carbon monoxide/oxygen inhibited the oxygen consumption of the developing insects by approximately 50 per cent.

7. *The effects of carbon monoxide on the post-injury metabolism of diapausing pupae*

In addition to the increased metabolism which accompanies the onset of adult development, the pupa during diapause can undergo a substantial increase in its metabolism under certain experimental conditions. Thus, after small localized injury to the pupal integument, the oxygen consumption and carbon dioxide production are considerably enhanced for one to several weeks thereafter (Sussman, 1952; Schneiderman and Williams, 1953a). This result has been regularly observed in both normal pupae and in pupae immobilized by prior removal of the abdominal ganglia.

Experiments were performed to test the sensitivity of the injury metabolism to carbon monoxide. To this end, the brains and abdominal ganglia were removed from six diapausing pupae. Two months later the rate of oxygen consumption was determined for each animal. A V-shaped 4-mm. incision was then made in the thoracic tergum of each animal, and the rate of oxygen consumption measured one day later. Three of the pupae were then placed in air-filled respirometers, compressed with five atmospheres of carbon monoxide, and the measurements repeated.

TABLE IV

The effects of carbon monoxide on the injury-stimulated respiration of brainless diapausing pupae lacking abdominal ganglia and connectives

		Rate of oxygen consumption* (%)					
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Prior to injury	In air	100	100	100	100	100	100
24 hours after injury	In air	158	208	108	198	146	146
30 hours after injury	In air	153	183	118	—	—	—
	In CO	—	—	—	183	152	128
Percentage difference between 1st and 2nd post-injury measurements		−3	−12	+9	−8	+4	−12

* Initial pre-injury oxygen consumption varied from 91 to 143 mm.³/animal/hour.

The respiration of the other three pupae was again measured in air at one atmosphere. The results recorded in Table IV show that the extra respiration stimulated by injury is *uninhibited* by carbon monoxide.

8. *Effects of cyanide on the respiration of diapausing pupae*

Diapausing pupae were injected with specific concentrations of cyanide and their oxygen uptake then ascertained. A typical set of measurements is plotted in Figure 5. In control experiments in which distilled water was injected, the oxygen uptake began to increase about five hours after the injection, and the typical pattern of injury metabolism became apparent. When cyanide was injected

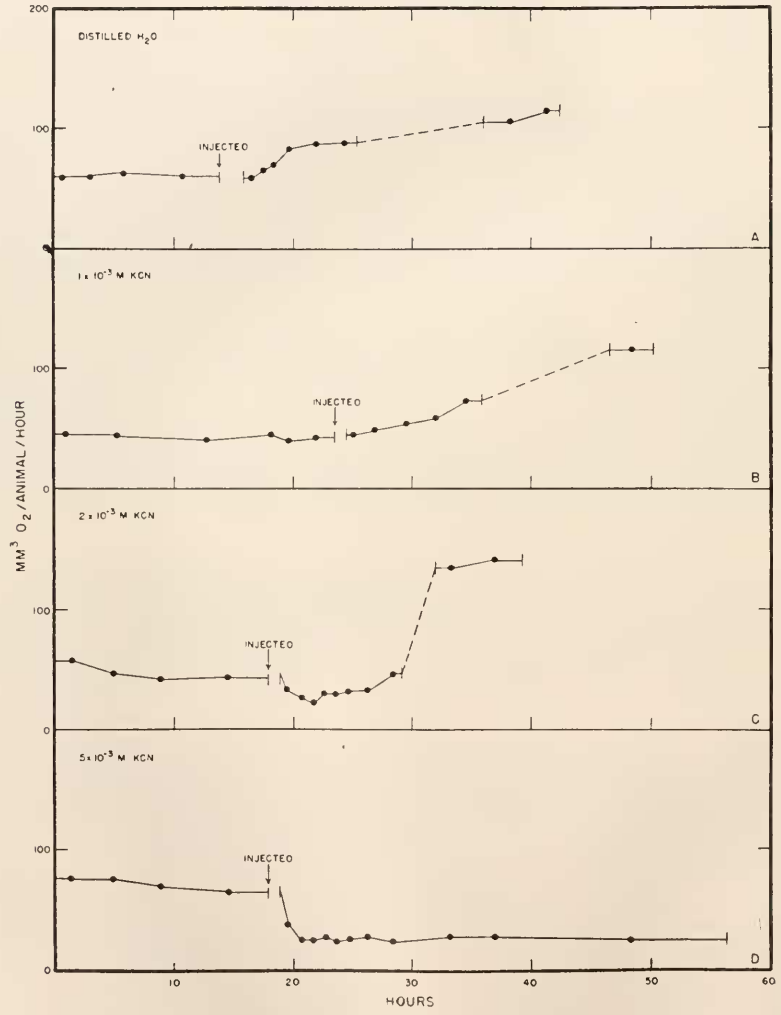

FIGURE 5. The effects of cyanide injection on the oxygen consumption of diapausing pupae. Concentrations of cyanide refer to calculated final internal concentrations.

to attain a final internal concentration of 10^{-3} M, a slight inhibition was usually observed, though in some cases the effect did not differ from that of distilled water. With further increase in cyanide to a final concentration of 2×10^{-3} M, an immediate inhibition of 50 per cent always occurred which persisted at a steady level for about ten hours and then gradually returned to normal. Still higher concentrations of cyanide (5×10^{-3} M and above) caused a prompt inhibition of 70 to 90 per cent, followed by the death of the animal several days later.

In the interpretation of experiments of this type it is necessary to separate the effects of injury from those of cyanide. This can most easily be accomplished by

TABLE V

The effects of cyanide on the respiration of diapausing pupae

Calculated internal concentration of HCN	Prior to injection		After injection	
	Average rate of oxygen consumption (mm.³/gm. live wt./hr.)	Lowest hourly rate of oxygen consumption as per cent of average initial rate*	Lowest hourly rate of oxygen consumption in 48-hour period as per cent of average initial rate	Behavior
0	10.7	97	98	Normal
	11.4	87	96	
	15.8	79	104	
	17.4	92	108	
	Average	89	102	
5×10^{-4} M	9.9	92	80	Normal
	10.3	96	102	
	10.5	88	97	
	12.1	91	89	
	12.5	94	98	
	Average	92	93	
5×10^{-4} M (Exposed to HCN gas for 60 hours but not injected)	8.1	95	93	Normal
	9.1	95	91	
	11.0	91	98	
	13.0	97	87	
	13.5	90	96	
	14.7	93	76	
	Average	93	90	
1×10^{-3} M	8.5	87	110	Normal, or slight decrease in abdominal motility
	9.7	95	107	
	11.4	88	94	
	11.4	94	51	Conspicuous decrease in abdominal motility for 12 to 24 hours
	10.5	83	85	
	12.2	100	72	
	28	93	67	
	Average	91	84	

* Measured during a four- to eight-hour period prior to cyanide injection.

TABLE V (*Continued*)

Calculated internal concentration of HCN	Prior to injection		After injection	
	Average rate of oxygen consumption (mm.³/gm. live wt./hr.)	Lowest hourly rate of oxygen consumption as per cent of average initial rate*	Lowest hourly rate of oxygen consumption in 48 hour period as per cent of average initial rate	Behavior
2×10^{-3} *M*	8.1	95	53	Electrically inexcitable for one day. Spontaneous abdominal movements reappeared after 2 days
	11.0	91	50	
	13.0	97	52	
	Average	94	52	
5×10^{-3} *M*	9.1	96	39	Died
	13.5	90	13	
	14.7	93	36	
	Average	93	29	
1×10^{-2} *M*	11.9	90	21	Died
	13.7	97	19	
	20.2	93	26	
	Average	93	22	

focusing attention on the oxygen uptake in the first few hours after injection and prior to the onset of the injury metabolism. As a measure of cyanide inhibition we chose the *lowest oxygen uptake* measured over a one hour interval during this period. To prevent any normal hour-to-hour variations in oxygen uptake from being interpreted as cyanide inhibition, we also recorded the *lowest hourly rate* of oxygen consumption during a four- to eight-hour period prior to cyanide injection. Table V summarizes such calculations on a series of 31 diapausing pupae. Detectable inhibition of abdominal motion and of respiration appeared only when the internal cyanide concentration, as calculated, was increased to or above 10^{-3} *M*.

In the evaluation of these findings it is worth recalling a technical difficulty mentioned in the section on Methods; namely, that it was impossible by the use of KCN–KOH mixtures to establish HCN concentrations higher than 5×10^{-4} *M* in the air surrounding the insect. Consequently, at internal concentrations higher than 5×10^{-4} *M*, detoxification and unspecific combinations of the injected HCN, along with the loss of HCN by diffusion from the tracheal system, necessarily reduced the internal concentrations below the calculated values. This fact complicated a decision as to the cyanide-sensitivity of the abdominal muscles. However, in experiments of short duration performed on a total of 18 diapausing pupae, we found that 10^{-3} *M* cyanide uniformly impaired the contractility of the abdominal muscles of normal pupae and totally eliminated the contractility of pilocarpine-stimulated muscles. In the latter case the muscles no longer responded to electrical stimulation. Consequently, we conclude that the carbon monoxide-sensitive abdominal muscles of diapausing pupae are likewise cyanide-sensitive.

Warburg (1949) emphasizes the fact that cyanide is a specific inhibitor of heavy metal enzymes only at concentrations up to about 10^{-3} M; above this level cyanide undergoes significant combinations with a large number of substrates and metabolic intermediates. In the case of Cecropia we attribute the lethal effects of cyanide concentration of 5×10^{-3} M and above to these unspecific side-reactions of cyanide.

9. *Effects of cyanide on the respiration of developing adults*

After the termination of diapause and the initiation of adult development, the effect of cyanide was easier to ascertain by virtue of the absence of injury metabolism, the latter being peculiar to diapausing pupae (Schneiderman and Williams, 1953c). Table VI summarizes the inhibition by cyanide of the respiration of 19

TABLE VI

The effects of cyanide on the respiration of developing adults

Calculated internal concentration of HCN	Days after initiation of adult development	Average rate of oxygen consumption prior to injection (mm.³/gm. live wt./hr.)	Oxygen consumption as per cent of average initial oxygen consumption at intervals after injection					Behavior after injection
			1.5 hrs.	8.5 hrs.	20 hrs.	26 hrs.	40 hrs.	
10^{-4} M	3	69	(124)	(66)			(110)	Emerged
	9	119	79	52			122	Emerged
	12	136	83	41			124	Emerged
	15	166	70	39			54	Died
	17	279	(92)	(20)			(30)	Died
		Average	77	44			100	
2×10^{-4} M	10	112	90		5	5		Died
	12	114	68		7	7		Died
	12	140	67		6	6		Died
	13	186	63		5	5		Died
	14	206	69		12	12		Died
		Average	71		7	7		
5×10^{-4} M	9	95	57		4		6	Died
	10	100	52		6		7	Died
	10	118	43		25		6	Died
	11	128	41		10		7	Died
	13	160	42		12		7	Died
		Average	47		11		7	
10^{-3} M	8	110	27		6		7	Died
	11	150	21		7		6	Died
	12	131	30		9		7	Died
	13	162	23		9		7	Died
		Average	25		8		7	

Figures in parenthesis () were not included in the average since these animals were not at comparable stages of adult development.

developing adults. The results have been averaged for animals between the 8th to 15th day of adult development. In contrast to the findings on diapausing pupae, cyanide at final concentrations of 2×10^{-4} *M* or higher was lethal; moreover 10^{-4} *M* cyanide now inhibited the oxygen uptake 35 to 80 per cent. In Table VI it also appears that the proportion of total metabolism which was cyanide-sensitive undergoes definite increase during the course of the twenty-two day period of adult development.

10. *Effects of oxygen tension on the respiration of brainless diapausing pupae lacking abdominal ganglia*

The experimental results, up to this point, demonstrate that systematic changes occur in the insect's dependency on metabolism sensitive to carbon monoxide and to cyanide. Aside from the intersegmental muscles of the abdomen, the metabolism of the diapausing pupa and the extra metabolism which it exhibits in response to injury are substantially insensitive to carbon monoxide and cyanide; by contrast, the metabolism of the developing insect is markedly inhibited by these agents.

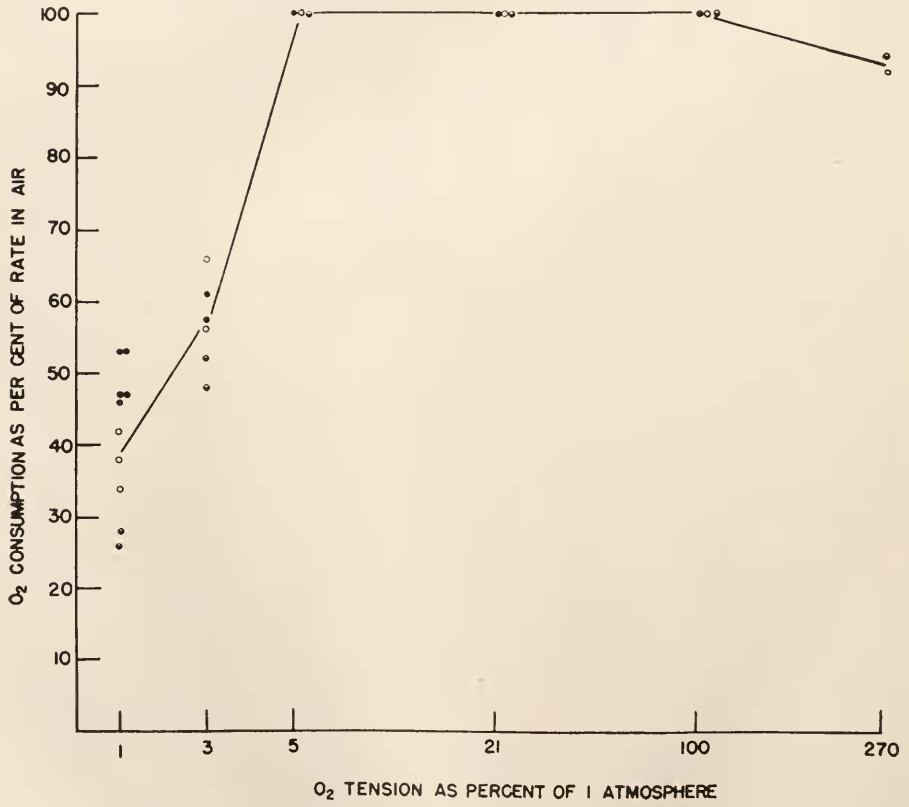

FIGURE 6. The effects of oxygen tension on the oxygen consumption of three brainless diapausing pupae lacking abdominal ganglia and connectives. The abscissa is marked off in arbitrary units.

Carbon monoxide-stable respiration has generally been found to require oxygen tensions far higher than does carbon monoxide-sensitive respiration (see Discussion). For this reason the effect of oxygen tension was studied in relation to the metabolism of diapause and development.

The oxygen consumption of three brainless diapausing pupae, previously immobilized by the removal of their abdominal ganglia, was determined at a series of oxygen tensions ranging from 1 to 270 per cent of an atmosphere of oxygen. The results of measurements at six different oxygen tensions are recorded in Figure 6. Each individual determination represents a steady-state value obtained after exposure to the gas mixtures for several hours. After each determination the pupae were returned to air for three days. The respiration in air was then re-determined before the pupae were exposed to a new oxygen tension.

As Figure 6 indicates, the oxygen consumption was independent of oxygen pressure at tensions from 5 per cent to 100 per cent of an atmosphere. In 3 per cent oxygen a conspicuous decrease was evident. But even in one per cent oxygen the average oxygen consumption was still 40 per cent of that in air. At the extremely high oxygen tension of 2.7 atmospheres, there appeared to be a slight depression, presumably attributable to the toxic effect of oxygen (Williams and Beecher, 1944).

Four pupae were stored in one per cent oxygen for ten hours. When returned to air, the oxygen consumption increased approximately 25 per cent above the normal rate in air and persisted at this level for several hours. This observation suggests the accumulation of a small but definite oxygen debt at the low oxygen tension. It is clear, however, that diapausing pupae possess limited ability to accumulate an oxygen debt for, as demonstrated in numerous experiments, several days of exposure to 0.5 per cent oxygen is lethal (L. D. 50% = 3 days at 25° C.).

Discussion

1. *Insensitivity of insects to compression*

Interpretation of the experimental results obviously requires a decision as to whether the experiments at high gaseous pressures were complicated by unspecific or narcotic effects of pressure *per se* (Behnke, 1940; Lawrence *et al.*, 1946).

As far as nitrogen is concerned, narcotic effects have not been demonstrated in any experiments on insects. Chadwick and Williams (1949) found that *Drosophila* could fly in 4.5 atmospheres of nitrogen plus one atmosphere of air, although the wingbeat frequency was decreased because of the increased gas density. Case and Haldane (1941) observed that *Drosophila* was active at 10 atmospheres of nitrogen plus one atmosphere of air, and Williams (unpublished) found that seven hours of exposure to 24 atmospheres of nitrogen plus one atmosphere of air failed to affect the vitality of *Drosophila* upon subsequent return to air. Moreover, experiments on the Cecropia silkworm at all stages of development, from egg to adult, have demonstrated that prolonged exposure to 6.7 atmospheres of nitrogen plus one atmosphere of air fails to retard embryonic or adult development, heart beat, movement, or the spinning of the cocoon (unpublished observations). And as demonstrated in the present study, the oxygen consumption was the same in air and in air compressed with five atmospheres of nitrogen. These results give assurance that nitrogen, at the pressures utilized, was not a narcotic.

We feel that the same conclusion is valid in the case of carbon monoxide. Thus, the oxygen consumption of immobilized diapausing pupae was the same in air and in air compressed with five atmospheres of carbon monoxide. Moreover, we shall subsequently show that many of the effects of high pressures of carbon monoxide on the post-diapausing insect are reversed by light (Schneiderman and Williams, 1954). Consequently, high pressure techniques appear to be useful and uncomplicated tools for experiments on insects.

2. Significance of carbon monoxide-sensitive respiration

As mentioned in the Introduction, suitable pressures of carbon monoxide are known specifically to inhibit the function of two enzymes, cytochrome oxidase and tyrosinase. Inhibition of cytochrome oxidase is reversed by light; inhibition of tyrosinase is not. Though the light-reversibility of carbon monoxide's action on post-diapausing Cecropia has already been described in the case of the male sex cells of Cecropia (Schneiderman, Ketchel and Williams, 1953) and will be considered in further detail at a later occasion, for our present purposes the phenomenon is doubly significant since it excludes tyrosinase as the critical target of carbon monoxide. This conclusion is further substantiated by the failure of phenylthiourea or any of a number of other potent anti-tyrosinases to duplicate the effects of carbon monoxide or of cyanide (Schneiderman and Williams, 1953a). We are therefore persuaded that the actions of carbon monoxide on Cecropia are due to its combination with cytochrome oxidase.

The factors which condition the quantitative effects of carbon monoxide on respiration mediated by the cytochrome oxidase system are four in number: (1) the relative affinity of the insect's cytochrome oxidase for carbon monoxide and for oxygen; (2) the carbon monoxide/oxygen ratio established at the site of enzyme action; (3) the degree to which cytochrome oxidase limits the transfer of electrons from substrate to oxygen; and (4) the oxidation of carbon monoxide to carbon dioxide. We shall briefly consider each of these points as it pertains to the present study.

Detailed measurements of the relative affinity of cytochrome oxidase for carbon monoxide and oxygen are available only in the case of yeast (Warburg, 1949) and mammalian heart muscle (Ball *et al.*, 1951). A 17:1 carbon monoxide/oxygen ratio inhibits the cytochrome-catalyzed respiration of yeast 75 per cent and the cytochrome oxidase activity of heart muscle 64 per cent. Since the relative affinities are so similar for such dissimilar cell types, it is a reasonable presumption that the insect cytochrome oxidase does not differ greatly. This probability has been confirmed by the finding that a carbon monoxide/oxygen ratio of 16:1 causes a light-reversible inhibition of the cytochrome oxidase activity of homogenates of the thoracic muscles of Cecropia moths by approximately 50 per cent (Pappenheimer and Schneiderman, unpublished observations).

In the positive pressure experiments on Cecropia a ratio of 25:1 was routinely established in the air surrounding the insect. The utilization of oxygen in the respirometer gradually lowered the oxygen tension over a period of 30 hours from 21 per cent to as low as 14 per cent, and thus increased the carbon monoxide/oxygen ratio. And, in each instance, the utilization of oxygen in the tissues decreased the internal oxygen tension still further. Consequently, the recorded ratio of 25:1 is

a minimal estimate of the effective carbon monoxide/oxygen ratio that was actually established in the insect's tissues at the site of enzyme action. For these several reasons we conclude that carbon monoxide in the positive pressure experiments effectively inhibited a large proportion of the insect's cytochrome oxidase—probably not far short of 100 per cent.

As mentioned above, one might anticipate that an excess of cytochrome oxidase relative to cytochrome *c* and other electron donors would tend to camouflage the participation of cytochrome oxidase in the metabolism of diapause. However, it is worth recalling that carbon monoxide combines exclusively with reduced cytochrome oxidase; that is, with functional oxidase receiving electrons from cytochrome *c*. An excess of the oxidase would necessarily be present in the oxidized form and therefore incapable of combining with carbon monoxide.

Finally, there is circumstantial evidence that the oxidation of carbon monoxide to carbon dioxide was not a complicating factor in the present study. If such an oxidation occurred, as described in the case of frog muscle by Fenn and Cobb (1932) and Stannard (1940), the oxidation of each molecule of carbon monoxide would be recorded manometrically as 1½ molecules of oxygen consumed and one molecule of carbon dioxide produced. The theoretical R.Q. of this process is 0.66, and in the case of the Cecropia pupa would thus tend to *decrease* the normal R.Q. of 0.78. However, since as recorded in Figures 2 and 3, a slight *increase* in R.Q. was actually observed in the presence of carbon monoxide, the oxidation of carbon monoxide was not a serious complication in the present experiments.

Thus, in summary, the conclusion seems acceptable that metabolism insensitive to high ratios of carbon monoxide/oxygen signals the utilization of terminal oxidases other than cytochrome oxidase.

3. *Significance of cyanide-insensitive respiration*

Cyanide is a far less specific inhibitor of cytochrome oxidase than is carbon monoxide. It inhibits not only cytochrome oxidase, but also catalase, peroxidase, and tyrosinase, and, as previously mentioned, at concentrations higher than about 10^{-3} *M*, cyanide also combines with various substrates, metabolic intermediates, and enzymes possessing carbonyl groups. For this reason cyanide-sensitivity is far less significant than cyanide-insensitivity. Cyanide-insensitivity strongly suggests that neither cytochrome oxidase nor any of a number of other enzymes are prerequisite for the reaction in question.

4. *Respiration during diapause and development*

As judged by its insensitivity to cyanide and carbon monoxide, virtually all of the metabolism of the diapausing pupa appears to proceed *via* pathways independent of cytochrome oxidase, save for the metabolic events responsible for the contraction of the abdominal muscles. It is therefore of particular interest and importance that the intersegmental muscle of the abdomen is the only major tissue within the diapausing pupa containing a high concentration of the classical cytochrome system (Williams, 1951; Pappenheimer and Williams, 1952).

The termination of diapause and the onset of adult development, however, usher in a new situation in which a progressively larger fraction of metabolism becomes dependent on the presence of a functional cytochrome oxidase system. Evidently,

at this time, a carbon monoxide- and cyanide-sensitive respiration mediated by cytochrome oxidase is superimposed on the carbon monoxide- and cyanide-stable metabolism of diapause.

Analogous changes in the relative activities of carbon monoxide-sensitive and carbon monoxide-stable respiratory systems have been demonstrated in a variety of plants, animals, and micro-organisms (Bodine and Boell, 1934a, 1934b; Wolsky, 1943, 1949; Paul, 1951). In the case of Cecropia the shift to cytochrome oxidase-mediated respiration is synchronized with the action of the insect's "growth and differentiation hormone" in terminating the pupal diapause—a correlation which suggests that the change in metabolism, in itself, is a part of the biochemical action of the hormone.

5. *Effects of oxygen tension on the respiration of brainless diapausing pupae*

The experimental results demonstrate that the metabolism of diapausing Cecropia pupae becomes independent of oxygen tension when the latter is five per cent of an atmosphere or higher. The tension of oxygen is usually considered to limit respiration at the cellular level only when it approximates zero (Krogh, 1916; Oppenheimer, 1925); this consideration is valid in most organisms since cytochrome oxidase, the usual terminal oxidase, is saturated by oxygen at tensions ranging from 0.25 to 2.5 mm. Hg (Winzler, 1941). However, flavoproteins when functioning as terminal oxidases are ordinarily thought to be unsaturated at low oxygen tensions. Thus, the "old yellow enzyme" which Warburg and Christian (1932) isolated from yeast was markedly influenced by variations in oxygen tension: in 100 per cent of an atmosphere of oxygen the respiration which it mediated was nearly five times that in 5 per cent oxygen. A corresponding dependency on oxygen tension has also been observed for flavoprotein-mediated respiration *in vivo*. Thus, in thin sections of the *Arum* spadix (James and Beevers, 1950), flavin-catalyzed oxygen uptake increased progressively as the oxygen tension was raised to one atmosphere.

The fact that the respiration of diapausing pupae is independent of oxygen at tensions greater than 5 per cent of an atmosphere, and the further fact that one per cent oxygen sustains 40 per cent of the normal respiration suggest that the carbon monoxide- and cyanide-stable oxidase of Cecropia differs from the flavoproteins reported in plants and bacteria and studied *in vitro*. However, we cannot exclude the possibility that such an oxidase may be present in relative excess in Cecropia and that it may fail to limit electron transmission even when driven slowly at low oxygen tensions.

6. *Identification of the terminal oxidases mediating respiration in diapausing pupae*

The results of the present study permit the following characterization of the terminal oxidases in diapausing Cecropia pupae. The principal terminal oxidase in the intersegmental muscles of the abdomen is cytochrome oxidase; in other major tissues of the diapausing insect it is not cytochrome oxidase. The latter unknown oxidase is uninhibited by high concentrations of carbon monoxide (carbon monoxide /oxygen ratios of 25:1), or by cyanide concentrations up to 10^{-3} *M*, or by phenylthiourea. Moreover, the oxidase in question is saturated by oxygen tensions of 5 per cent of an atmosphere or less.

On the basis of these several lines of evidence, the most probable candidates appear to be either an autoxidizable flavoprotein transferring electrons from reduced pyridine nucleotides to molecular oxygen, or an autoxidizable heme-containing enzyme which fails to combine with either cyanide or carbon monoxide.

Summary

1. The respiration of the Cecropia silkworm was studied after the injection of cyanide or in the presence of specific mixtures of oxygen, nitrogen, and carbon monoxide. Positive pressure techniques were utilized to test the effects of carbon monoxide/oxygen ratios as high as 25 : 1.

2. It was found that the respiration of the diapausing pupa is only slightly affected by high concentrations of carbon monoxide or cyanide. This minor effect was accounted for in terms of the cyanide- and carbon monoxide-sensitivity of the contraction of the intersegmental muscles of the pupal abdomen. The other tissues in the dormant insect showed no detectable inhibition by high concentrations of cyanide or carbon monoxide.

3. The termination of the pupal diapause and the progress of adult development are accompanied by a marked increase in the insect's sensitivity to cyanide and carbon monoxide. The effects of these agents are then no longer limited to muscular tissue but extend to the insect as a whole. Cyanide or carbon monoxide appear to act exclusively on the extra metabolism accompanying development and, thereby, to reduce the overall metabolism to the old diapausing level.

4. The modes of action of cyanide and carbon monoxide within the diapausing and non-diapausing insects are considered in detail. Insensitivity to these agents, as in most tissues of the diapausing pupa, argues in favor of the presence and utilization of a terminal oxidase other than cytochrome oxidase.

5. It is concluded that cytochrome oxidase is the principal terminal oxidase of only the somatic musculature of the diapausing pupa. Months later, with the termination of the pupal diapause, cytochrome oxidase becomes the principal terminal oxidase of the growing, post-diapausing insect as a whole.

6. These qualitative changes in the insect's metabolism are synchronized with the secretion of the hormone responsible for the termination of diapause and the development which follows, and appear to be a more or less immediate result of the hormonal action.

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